Free Radical Scavenging and Antibacterial Activities of *Helichrysum caespititium* (DC) Harv. Extracts

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**Abstract**

*Helichrysum caespititium* is a medicinal plant used in the Kingdom of Lesotho to treat head, chest colds, and during circumcision rites. The aim of this study is to evaluate the free radical scavenging capability of five extracts (*n*-hexane, dichloromethane, acetone, methanol, and water extracts) of *H. caespititium* against reactive oxygen species (ROS) and reactive nitrogen species (RNS), and their reducing power and their antibacterial activities. Qualitative and quantitative free radical scavenging activities of the extracts were evaluated using bioautography antioxidant assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (RNS) free radical scavenging, hydrogen peroxide (ROS) free scavenging, and reducing power assays. In the study, all the extracts were tested against 14 bacterial strains that are both Gram positive and Gram negative suspended in Mueller-Hinton Broth. Qualitative antioxidant evaluation of all the extracts by DPPH showed that all five extracts exhibit some antioxidant compounds, and the quantitative results provided the DPPH scavenging activity of 94.3% at 0.4 and 0.5 mg/ml for acetone extract (*IC*$_{50}$ = 0.055 mg/ml), 91.2% inhibition of hydrogen peroxide at 0.5 mg/ml for water extract (*IC*$_{50}$ = 0.075 mg/ml), and a reducing power of 62.3% at 0.4 mg/ml for *n*-hexane extract (*IC*$_{50}$ = 0.092 mg/ml). The reducing power of *n*-hexane and the DPPH scavenging ability of acetone extracts were better than ascorbic acid, but less than butylated hydroxytoluene. Ascorbic acid was a slightly better scavenging hydrogen peroxide compared to water extract, both at 0.4 and 0.5 mg/ml. All the bacteria both Gram positive and Gram negative were susceptible to all the extracts, and the minimum inhibitory concentration values of the extracts were below 0.4 mg/ml. These findings justify the utilization of the whole plant in the traditional sector as antioxidant, and the plant could be a better remedy against a broad range of bacteria as well as a good crude natural antioxidant agent.

**Keywords**: Antioxidant; Antibacterial; *Helichrysum caespititium*; Minimum inhibitory concentration

**Introduction**

Several scientists have reported that the genus *Helichrysum* exhibits antimicrobial activities [1-6]. However, in all these, little is depicted regarding *Helichrysum caespititium* species.

*Helichrysum caespititium* (DC) Harv. (*H. caespititium*) is a prostate, perennial, mat-forming herb that is profusely branched and densely tufted. The leaves are dotted with orange glands. The flowers are silvery white with yellow centers and a pale furry underneath. The plant flowers in the late summer.

The plant is used to treat nausea and virility [7]; the plant decoction is drunk by Bakwena and Bakgatla from South Africa in the treatment of gonorrhea [7]. In addition, Basotho in Qwaqwa in the Free State province is drunk by Bakwena and Bakgatla from South Africa in the treatment of liver damage and acting as carcinogens in laboratory animals [13]. These were the subjects of this research.

In order to obtain a more complete insight into the antibacterial activity of the plant and its free radical scavenging ability, different solvents (*n*-hexane, dichloromethane, acetone, methanol and water) were used to extract the phytochemicals. Moreover, the scavenging activity of the extracts was measured using DPPH, hydrogen peroxide, and reducing power. The selection of the solvents was guided by their polarity, from nonpolar solvents (*n*-hexane), midpolar solvents (dichloromethane and acetone) to high-polar solvents (methanol and water).

**Materials and Methods**

**Plant material**

The plant was botanically identified by the South African National Biodiversity Institute (SANBI) under the specimen number DTH 9006000 in the Pretoria herbarium, Gauteng Province, South Africa. The plant was collected from Masealama village (situated at 23.8346°S, 29.8844°E) in Capricorn District Municipality in the Limpopo province, South Africa. After collection, the plant material was cut into small pieces and dried at 25°C for a period of approximately two months. Thereafter, the dried plant material was pounded using Retsch cutting mills SM 100 machine, resulting into a fine powder. The powder was kept until usage.
Extraction methods

n-Hexane, dichloromethane, acetone, methanol, and water were used to extract secondary metabolites from the whole plant [14,15]. The parts of the plant were not distinguished, because it was not easy to separate them.

Briefly, 160 g of powdered plant was macerated at room temperature in 5000 ml of each solvent successively for 24 h on a shaker (Labotec 262, South Africa) at 150 rpm. The process was repeated three times using the same volume of fresh solvent with the same plant material by each solvent to make sure that most of the secondary metabolites are extracted. The mixture was settled down before being filtered using Whatman No. 1 filter paper before solvent evaporation using a rotary evaporator under reduced pressure at 20–40°C. The filtrates were transferred into preweighed beakers and then ventilated to dryness.

Antioxidant activity test

The antioxidant test was conducted qualitatively and quantitatively on the five plant extracts. The qualitative test (bioautography antioxidant assay) involved DPPH only, and the quantitative test involved DPPH, hydrogen peroxide, and reducing assays.

DPPH radical scavenging assay

First, the antioxidant qualitative assay was performed on thin layer chromatography (TLC) (Figure 1) using different solvents system (benzene–ethanol–ammonia, BEA; ethyl acetate–methanol–water, EMW, and chloroform–ethanol–formic acid, CEF) to determine whether there are antioxidant compounds in the five plant extracts.

The TLC using these different solvent systems were dried after being sprayed with 0.2% solution of DPPH free radical. The reduction of DPPH in the presence of an antioxidant agent changes its color from purple-red to yellow (Figure 1).

Second, the quantitative DPPH assay was performed following the method described by Olivier et al. [16]. The mixture of extract solutions and DPPH were incubated in the dark for 30 min. Subsequently, the absorbance was read using a spectrophotometer at 517 nm. Ascorbic acid and BHT methanolic solutions were used, respectively, as standard and methanolic solution of DPPH free radical as negative control. The percentage of radical scavenging activity (Graph 1) was calculated using the following formula.

\[
\% \text{DPPH radical scavenging activity} = 1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{sample}}\) and \(A_{\text{control}}\) are the absorbance of the sample and the control, respectively.

Hydrogen peroxide scavenging assay

The ability of the extracts (n-hexane, dichloromethane, acetone, methanol, and water) of Helichrysum caespititium to scavenge hydrogen peroxide was conducted using the method as described by Olivier et al. [16]. Twenty millimolar (20 mM) of hydrogen peroxide solution was prepared from phosphate buffer saline (PBS) with a pH 7.40. The extracts were previously suspended in 1 ml of DMSO before preparing their solutions at different concentrations 0.09, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml in water. A volume of 2 ml of hydrogen peroxide in PBS were mixed with 1 ml of plant extract solution. The mixture was vortexed, and then incubated for 10 min before measuring the absorbance at 230 nm. The blank solution contained PBS without hydrogen peroxide. The percentage inhibition was calculated by using the following equation, and the results are provided in Graph 2. Ascorbic acid was used as a standard.

\[
\% \text{of } H_2O_2 \text{ inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{sample}}\) and \(A_{\text{control}}\) are the absorbance of the sample and the control, respectively.
Reducing power assay

The reducing power assay is used for evaluating the ability of plant extracts to reduce Fe3+ ions to Fe2+ by electron donation. This was facilitated by the presence of potassium ferricyanide (K3[Fe(CN)6]). The analysis was conducted using the protocol as described by Oyaizu [17] with minor modifications. Briefly, plant extracts were diluted into 1 ml of DMSO, and then diluted with distilled water into 25 ml resulting to a stock solution of 1.0 mg/ml. The working plant extract solutions were prepared by diluting the stock solution to 0.09, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml. One milliliter (1.0 ml) of the working solution of the plant extract was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.60) and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide (K3[Fe(CN)6]). The mixture was vortexed, and then incubated in water bath at 50°C for 20 min. Thereafter, 2.5 ml of a 10% (w/v) trichloroacetic acid (C2HCl3O2) was added, and then centrifuged at 3000 rpm for 10 min. A volume of 2.5 ml of the upper layer was transferred into a test tube before being mixed with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride (FeCl3). The mixture was well vortexed before the absorbance was measured at 700 nm using the spectrophotometer. The increase in the absorbance of the reaction mixture indicated the increased reducing power of plant extracts at different concentrations. Ascobic acid and BHT were used as standards. The percentage inhibition was calculated by using the following equation:

\[
\% \text{ of reducing power} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \( A_{\text{sample}} \) and \( A_{\text{control}} \) are the absorbance of the sample and the control, respectively.

Microorganisms

The evaluation of the antibacterial activities was following a protocol published by Andrew [18], and this was conducted against 14 pathogenic strains both Gram-positive and Gram-negative strains: Bacillus cereus (ATCC10876), Bacillus subtilis (ATCC19659), Enterococcus faecalis (ATCC13047), Mycobacterium smegmatis (MC2155), Staphylococcus epidermidis (ATCC14990), Escherichia coli (ATCC25922), Enterobacter cloacae (ATCC13047), Klebsiella oxytoca (ATCC8724), Klebsiella pneumonia (ATCC13882), Proteus vulgaris (ATCC6380), Pseudomonas aeruginosa (ATCC27853), Proteus Mirabilis, Staphylococcus aureus, and Enterobacter aerogenes. The stock solution (0.8 μg/ml) of each extract was prepared in DMSO or water, and 100 μl of each of them were serially diluted (1:1) to give 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 mg/ml and were seeded in 96-well plates. An overnight fresh culture (50 μl) containing 1.5 × 10^5 cfu/ml of each strain in Mueller-Hinton Broth was seeded in 96-well plates containing 50 μl different concentrations in duplicate to make a final volume of 100 μl and allowed to grow overnight with 5% CO2 flow at 37°C. Streptomycin (STM) and Nalidixic acid (NLD) were used as controls. The viability of cells was confirmed using resazurin dye after 5 h incubation, and the MIC was recorded for each extract.

Results and Discussion

The bioautography assay has shown that the whole plant extract exhibits antioxidant compounds, and their intensity is shown by the multitude of the yellow spots on the TLC for a particular extract.

The free radical scavenging activities of H. caespititium extracts (quantitative assay) were evaluated by DPPH, hydrogen peroxide, and reducing power assays in comparison with ascobic acid and BHT as standards (Graphs 1, 2, and 3). Moreover, their IC50 values are depicted in Table 1. Ascobic acid exhibits a high activity from a concentration of as little as 0.09 mg/ml compared to others. The radical scavenging activity of all extracts of H. caespititium plant against DPPH is observed from 0.09 mg/ml; however, the highest activity was observed from the same concentration for all the extracts with a low IC50 of 0.05 mg/ml.

The radical scavenging ability of all the extracts of the plant on DPPH was strong comparable to one of the standard (ascorbic acid) and close to the other (BHT). This suggests that there are some antioxidant compounds in the plant that can be isolated using even a nonpolar solvent (n-hexane). This is contrary to what one could expect. This is because the extraction of antioxidant compounds from a plant is more related to the polarity of the solvent used [19]. Therefore, one has to determine the classes of secondary metabolites that are present in the n-hexane extract and that are responsible for the antioxidant activity observed in the extract.

The whole plant is strongly scavenging DPPH at % inhibition above 91%. In some instance, the extracts (acetone and water extracts) exhibit the highest % inhibition of DPPH from the concentration of 0.3 to 0.5 mg/ml than one of the standard (ascorbic acid). These suggest that the plant can substitute ascobic acid as an antioxidant in places where there is scarcity of it.
We observed that the radical scavenging ability of the whole plant was strongly against hydrogen peroxide with a low IC$_{50}$ of 0.075 mg/ml. Ascorbic acid showed more activity against hydrogen peroxide compared to the strongest plant extract (water). In general, the whole plant exhibits a strong scavenging activity of below 0.37 mg/ml. The results for both DPPH and hydrogen peroxide are encouraging, because it is known that the traditional healers use water to extract ingredients from the plant. Moreover, it shows that the traditional healers extract more of antioxidant using water that is able to scavenge both DPPH and hydrogen peroxide. Although the plant is a strong reactive nitrogen species (RNS) and reactive oxygen species (ROS) radical scavenger, it is more of the RNS scavenger than ROS. There is strong evidence that a mechanistic link involves the generation of ROS and RNS by macrophages and neutrophils that respond to cytokines and other signaling processes arising at sites of inflammation. These ROS cause oxidation, nitration, halogenation, and deamination of biomolecules of all types, including lipids, proteins, carbohydrates, and nucleic acids, with the formation of toxic and mutagenic products [20-22].

This suggests that *H. caespititium* can be used against inflammation. It explains the usage of this plant as a wound dressing material in male circumcision rites, and for “internal wounds” [23]. Moreover, it can be used to cure sperm motility problem on asthenozoospermic patients, because a critical negative effect of peroxynitrite (a RNS) on sperm motility was observed when spermatozoa concentration was normal [21]. Thus, a possible pathogenic role in infertile men when asthenozoospermia is the main critical problem may be cured; however, this should be proven scientifically.

Tentatively, these results shed some light on why the plant is used to treat virility [7]. n-hexane extract showed a very good reducing power above 50% almost across all the concentrations used. Moreover, 62.3% of reducing power on ferric ion (Fe$^{3+}$) (IC$_{50}$ = 0.092 mg/ml) by n-hexane extract at 0.4 mg/ml. From this observation, one could conclude that there is a strong electron transfer from the plant extract to reduce Fe$^{3+}$ to Fe$^{2+}$. It is known that radicals destroy human body by reacting with some human compartment such as DNA and also initiate lipid peroxidation that causes DNA damage in the body [22].

These results reveal that the plant can be used against all the disease for which oxidants are responsible for their prevalence. All five plant extracts tested were active against both Gram-positive and Gram-negative bacteria with the MIC values ranging from 0.4 to 0.01 mg/ml (Table 2).

According to Gibbons et al. [23], the values of MIC below 1 mg/ml for extracts and 64 µg/ml for single chemical entities are considered significant. Therefore, these results (Table 2) are worth considering. Two Gram-positive bacteria were inhibited more by the extracts than others. *Enterococcus faecalis* was observed to be inhibited more by three plant extracts: water extract (MIC = 0.02 mg/ml), hexane extract

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>Gram +/−</th>
<th>MIC (mg/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HE</td>
<td>DE</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>+</td>
<td>0.2</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>+</td>
<td>0.1</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>+</td>
<td>0.02</td>
</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>+</td>
<td>0.1</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>–</td>
<td>0.4</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>–</td>
<td>0.1</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>–</td>
<td>0.1</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>–</td>
<td>0.02</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>–</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Minimum inhibitory concentration; HE, hexane extract; DE, dichloromethane extract; AE, acetone extract; ME, methanol extract; WE, water extract.

<table>
<thead>
<tr>
<th>Extracts and Standards</th>
<th>DPPH (IC$_{50}$)</th>
<th>Hydrogen peroxide (IC$_{50}$)</th>
<th>Reducing power (IC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.E = n-hexane extract</td>
<td>0.062</td>
<td>0.2</td>
<td>0.092</td>
</tr>
<tr>
<td>D.E = dichloromethane extract</td>
<td>0.06</td>
<td>0.357</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>A.E = acetone extract</td>
<td>0.055</td>
<td>0.315</td>
<td>0.33</td>
</tr>
<tr>
<td>M.E = methanol extract</td>
<td>0.05</td>
<td>0.18</td>
<td>0.4</td>
</tr>
<tr>
<td>W.E = water extract</td>
<td>0.05</td>
<td>0.075</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>Ascorbic acid (Std)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.4</td>
</tr>
<tr>
<td>BHT (Std)</td>
<td>0.04</td>
<td>−</td>
<td>&gt; 0.5</td>
</tr>
</tbody>
</table>

*IC$_{50}$ (mg/ml): Inhibition concentration at which 50% of DPPH radicals and hydrogen peroxide are scavenged.

IC$_{50}$ (mg/ml): Inhibition concentration at which 50% of DPPH radicals and hydrogen peroxide are scavenged.
and dichloromethane extract (MIC = 0.01 mg/ml) was found to be inhibited by
three plant extracts: water extract (MIC = 0.01 mg/ml), acetone extract (MIC = 0.02 mg/ml), and hexane extract (MIC = 0.01 mg/ml). However, four Gram-negative bacteria species were inhibited more by
the plant extracts than others. Escherichia coli was found to be inhibited
more by two plant extracts: methanol extract (MIC = 0.02 mg/ml) and
dichloromethane extract (MIC = 0.01 mg/ml), Klebsiella oxytoca was inhibited by one extract: water extract (MIC = 0.01 mg/ml), and
Klebsiella pneumonia was also inhibited by one extract: dichloromethane
extract (MIC = 0.02 mg/ml). Proteus vulgaris was inhibited significantly
compared to other species by all the five extracts: acetone extract (MIC = 0.01 mg/ml), water extract (MIC = 0.02 mg/ml), methanol extract (MIC = 0.05 mg/ml), hexane extract (MIC = 0.02 mg/ml), and
dichloromethane extract (MIC = 0.01 mg/ml). The bacteria species
that were inhibited by the highest dilution (0.01 mg/ml) in this study are Enterococcus faecalis, Staphylococcus epidermidis, Escherichia coli, Klebsiella oxytoca, and Proteus vulgaris. H. caespititium whole plant looks promising against both Gram-positive and Gram-negative
bacteria species.

This plant exhibits a broad spectrum of activity with low MIC values starting from 0.5 to 0.01 mg/ml. The acetone extract was previously studied by Mathekga et al. [5], and similar strength of activity was observed across the many bacteria studied. Only Klebsiella pneumoniae and Serratia marcescens did not exhibit activity. In our case, we exposed Klebsiella pneumoniae against all the extracts; all the extracts inhibited the growth of Klebsiella pneumonia with the MIC values ranging from 0.4 to 0.02 mg/ml. The acetone extract used by Mathekga et al. [5] showed no activity. This discrepancy might be explained by the season at which the plant material was collected. It has been discovered by Gololo et al. [24] that season change can affect the phytochemical component of a plant. Mathekga et al. [5] collected their plant for study in August (late winter). Moreover, in this study, we collected our plant in February (midsummer); these two different plant collection periods might be the cause for the previous researcher to not observe any activity of the plant against Klebsiella pneumoniae. However, in our case, the activity was observed with the MIC value of 0.4 mg/ml. Most bacillus species are known to have little or no pathogenic potential; however, both Bacillus cereus and B. subtilis have been known to act as primary invaders or secondary infectious agents in a number of cases and have been implicated in some cases of food poisoning [25]. Staphylococcus aureus (a human pathogen) and Pseudomonas aeruginosa, whose infections are among the most difficult to combat with conventional antibiotics [2,26], were found to be inhibited by the H. caespititium extracts. This plant may, thus, be a source of drugs that can improve the treatment of infection caused by these microorganisms.

This finding provides a clear understanding of the usage of the whole plant by indigenous people to treat different human malaise. In light of the fact that Gram-negative bacteria are more resistance to antimicrobial agents from plants than Gram positive [27], this plant has a brighter future in terms of drug development.

Conclusion

In conclusion, this study managed to reveal some truth about the
plant antioxidant and antibacterial activities. The plant, in general,
is a strong antioxidant agent with n-hexane extract being the most comparable to ascorbic acid in potency. The plant can be used to
tackle the problem of infertility in men. This is because it showed

References


